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STATE-OF-THE-ART REVIEW

Antibodies gone bad – the molecular mechanism of light chain amyloidosis

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Light chain amyloidosis (AL) is a systemic disease in which abnormally proliferating plasma cells secrete large amounts of mutated antibody light chains (LCs) that eventually form fibrils. The fibrils are deposited in various organs, most often in the heart and kidney, and impair their function. The prognosis for patients diagnosed with AL is generally poor. The disease is set apart from other amyloidoses by the huge number of patient-specific mutations in the disease-causing and fibril-forming protein. The molecular mechanisms that drive the aggregation of mutated LCs into fibrils have been enigmatic, which hindered the development of efficient diagnostics and therapies. In this review, we summarize our current knowledge on AL amyloidosis and discuss open issues.

Introduction

Amyloidoses have been studied from the mid-1800s onwards [1]. In the 20th century, it became clear that amyloids can be formed by various proteins which deposit in fibrillar structures. The first suggestions that linked amyloids and immunoglobulin (Ig) light chains (LCs; referred to as Bence-Jones proteins when found in urine) can be tracked back to the work of Adolf Magnus-Levy almost a century ago [2,3]. Pioneering work by Elliot F. Osserman further established the correlation between plasma cell dyscrasia, Bence-Jones proteins and amyloidosis [4–6]. In the 1970s, it became evident that the antibody sequences found in amyloids from different patients are diverse [7]. The proteins deposited as amyloids were identified as Ig LCs and often their truncations, mostly comprising their variable domains (V_Ls) only [8,9]. *In vitro* it was shown that LCs subjected to proteolysis and low pH can form amyloids [10]. However, the discussion of whether LCs are

Abbreviations
AL, antibody light chain amyloidosis; Apo, apolipoprotein; BiP, binding immunoglobulin protein; Cath, cathepsin; CDR, complementarity-determining region; C_L, constant light chain domain; ECM, extracellular matirx; ER, endoplasmic reticulum; ERAD, ER associated degradation; ERQC, ER quality control; GL, germline; HC, heavy chain; HDM-SCT, autologous peripheral blood stem cell transplantation; Ig, immunoglobulin; IGKV, immunoglobulin kappa variable cluster; IGLV, immunoglobulin lambda variable cluster; LC, light chain; LCDD, light chain deposition disease; MDex, dexamethasone; MM, multiple myeloma; MMP, matrix metalloprotease; SAP, serum amyloid component P; SHM, somatic hypermutation; TIMP, tissue inhibitor of metalloprotease; UPR, unfolded protein response; V_L, variable light chain domain; κ, kappa; λ, lambda.

the major precursors that lead to amyloid formation continued for a few years [11]. Ultimately, it was shown that the Bence-Jones proteins and the amyloid fibril proteins of a patient are identical [12]. Large-scale studies further demonstrated that not all LCs isolated from patients form amyloids *in vitro* and that certain LC subtypes show a higher prevalence in antibody LC amyloidosis (AL) than others [13–17]. Eventually, databases were created in which information on thousands of different LC variants found in AL patients were deposited and clinical centers dedicated to the study and treatment of AL were established [18]. Thanks to significant advances over the last decades in understanding the biology behind the disease, a general picture of the molecular mechanisms is beginning to emerge. In this review, we will discuss structural aspects of fibrils, disease-causing mutations, the LC escape from the cellular quality control, the role of extracellular components and the sequence of events leading from the native antibody structure to fibrils.

AL – disease and treatment

Light chain amyloidosis is an acquired amyloidosis except for one reported hereditary mutation located in the constant region of the LC (S131C) [19,20]. The yearly incidence of AL was 8.9 and 12.5 cases per million people in two independent population-based studies [21,22] with a higher frequency in older individuals [22–24].

Light chain amyloidosis patients typically have acquired a monoclonal gammopathy and thus exhibit high serum concentrations of an amyloidogenic LC which may be comorbid with an atypical form of multiple myeloma (MM) [20,25,26]. The amino acid sequences of the overexpressed LCs are largely patient-specific and the symptoms vary making the diagnosis of AL complicated [20,27]. However, the early diagnosis of AL and the definition of organ involvement is crucial for successful therapy [25,28], also as the prognosis for AL patients depends on the organs involved [29–31]. In this context, cardiac involvement is related to poor prognosis [30,31] as the increasing fibril deposits lead to mechanical impairment of organ function. Other proposed mechanisms of AL-induced organ damage are cytotoxicity, generation of reactive oxygen species and metabolic dysfunction [32–35]. While the heart and kidney are most frequently affected, AL can also involve other organs. The reasons why LC amyloids target certain organs are still unclear. It was proposed that certain immunoglobulin lambda variable cluster (IGLV) germline gene segments pose a higher risk for cardiac (*IGLV1–44*) [36,37], renal (*IGLV6–57*) [38] or hepatic (*IGKV1–33*) [37] involvement.

Current therapeutic approaches are mainly aimed at suppressing LC synthesis (plasma-cell directed) while the development of new therapeutics that can remove amyloid deposits from affected organs is gaining focus (fibril-directed) [20,39]. In addition, supportive care to alleviate symptoms due to specific organ involvement is required. The current treatment of AL depends on the risk group of the patient [20]. AL patients at low risk can undergo chemotherapy with high doses of melphalan followed by autologous peripheral blood stem cell transplantation (HDM–SCT) to target the monoclonal gammopathy [40–42]. HDM–SCT can slow down or stop the progression of AL but does not remove existing fibril deposits. Some patients at intermediate or high risk can also undergo HDM-SCT, provided that they have partially preserved organ function [20]. A combination of melphalan and dexamethasone is used for the treatment of AL patients at intermediate risk [43]. Also, proteasome inhibitors like bortezomib are included in combination therapies for these patients [44]. The treatment of AL patients at high risk represents an outstanding challenge [20]. Heart, kidney or liver transplantation are treatment options for AL patients with severe organ involvement [45].

Clearly, additional treatment options are needed and there are several ongoing studies for novel therapies with proteasome inhibitors (carfilzomib [46] and ixazomib [47]), immunomodulatory drugs (pomalidomide [48]), anti-CD-38 antibodies (daratumumab [49] and elotuzumab [50]) or alkylating agents (bendamustine [51]). Since most of these therapeutics reduce the production of new amlyoidogenic LCs but cannot treat the existing organ damage, fibril-directed monoclonal antibodies (11-1F4 [52] and NEOD001 [53]) are tested as therapeutics to remove fibril deposits. Furthermore, the kinetic stabilization of LCs to prevent them from unfolding and aggregation – and thus fibril formation – came into focus [54].

Antibody and light chain structure

To understand the mechanisms of AL, it is important to consider the structure and diversity of LCs. The human Igs are hetero-tetrameric glycoproteins consisting of heavy chains (HCs) and LCs [55]. In its simplest version, Ig consists of two HCs and two LCs which are connected by disulphide bonds (Fig. 1A). The type of HC determines the antibody class – IgA, IgD, IgE, IgG or IgM. The LCs belong either to the λ or the κ family, whose encoding genes are located on

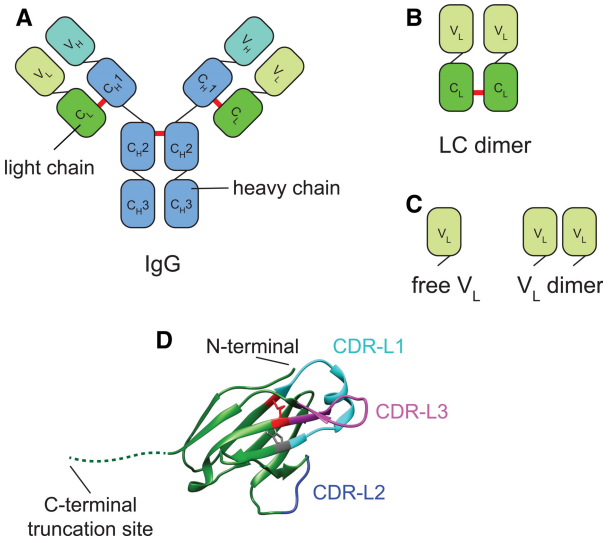

Fig. 1. Structural elements in antibodies. (A) An IgG antibody is composed of two identical LCs and two identical HCs; (B) LC dimer; (C) monomeric and dimeric LC V_L domains that occur from cleavage of LCs. Disulphide bonds are indicated by red lines. (D) Crystal structure of a V_L domain (PDB: 6SM2). The intramolecular disulphide bond is shown in red. The conserved tryptophan residue is highlighted in grey. The CDRs (Kabat definition) are in cyan (CDR-L1), blue (CDR-L2) and magenta (CDR-L3). The length of the sequence at the C-terminus can vary depending on the truncation site between the V_L and C_L. The structure has been generated using UCSF Chimera.

different gene loci [56]. Both LC families can pair with each of the HC classes. Both the HCs and LCs contain variable domains, V_H and V_L respectively, which are responsible for antigen-binding. In addition, they comprise constant domains (Fig. 1A). The LCs consist of two domains: the variable domain V_L and the constant domain C_L.

While the differences between the biological properties of the HC classes are well established [57], there is still a discussion about the benefits of LC variability. Each of the two LC types further comprises a number of subfamilies. The subfamilies can have multiple members depending on the number of different germline V gene segments, for example, there are three members of the λ IV subfamily, λ IVa/b/c [58–60] with structural differences in the complementarity-determining regions (CDRs) and the linker region [61,62]. The ratio of κ and λ LCs in the serum of a healthy person is approximately 2 : 1 [63]. Other species have vastly different physiological ratios between κ and λ LCs. For example, in cattle, 90–95% of the LCs are from the λ family, while in mice almost all LCs are from the κ family [64,65]. The importance of different ratios of κ and λ LCs in the serum is still enigmatic. In humans, a non-physiological κ to λ ratio can be an indication of underlying pathological conditions like myeloma [66]. Interestingly, in AL patients the λ family is overrepresented with a λ : κ-ratio of approximately 3 : 1 [67].

One of the hallmarks of AL amyloidosis is the high level of free LC monomers and homodimers in the serum and other body fluids of patients with MM or AL (Fig. 1B) [68,69]. Dimer formation is mediated by the hydrophobic interfaces that would normally participate in LC/HC contacts in antibodies as well as by intermolecular disulphide bridges via C-terminal cysteines. Especially the C_L–C_L interface provides stabilizing interactions in the LC dimer [70]. Moreover, it is known that the C_L domain can have a profound stabilizing effect on the V_L domain [71]. In the context of AL, proteolytic cleavage *in vivo* results in the release of free V_L domains that exist either as monomers or as homodimers (Fig. 1C).

The basis of LC sequence variability

While amyloid diseases share common characteristics, AL amyloidosis is set apart by one unique aspect: the vast sequence variability of the precursor protein [72] which is connected to the biological function of Igs, that is, binding to a large number of different antigen structures with high affinity during immune responses. The sequence variability of LCs is created during B cell development by recombination of variable and joining gene segments (V–J recombination) and somatic hypermutation (SHM) [73–76]. The most variable parts in LCs are the three loops of the CDRs (L1, L2 and L3) that form part of the antigen-binding site in functional antibodies (Fig. 1D).

In addition to the variability resulting from gene segment recombination, somatic mutations in the V_L domain can further increase the sequence space of LCs. In the case of AL amyloidosis, genetic aberrations due to the underlying plasma cell dyscrasia (e.g., in MM) introduce additional amino acid substitutions in LC sequences [77,78]. Together, these mutations play a key role in the onset and progression of the disease as they can affect LC folding and stability and therefore govern the transition from soluble precursor proteins to insoluble amyloid fibrils.

Fibril structure

Different proteins can be amyloidogenic, resulting in about 40 known amyloidoses [79–82]. The amyloids share a defining common feature – they are composed of highly ordered, long protein filaments or fibrils with

(mostly) cross-β sheet structure. Originally, amyloid plaques were defined by their ability to induce birefringence in tissue stained with Congo red [83]. Later on, it was discovered that the binding of the benzothiazole-based dye Thioflavin T to fibrils and aggregates with amyloid-like structure results in a change in its fluorescence emission and thus enables monitoring of amyloid fibril formation [84,85]. Fibrils also exhibit a characteristic X-ray diffraction pattern [86–88] as a defining feature. Detailed analyses of different amyloid fibrils using high-resolution structural techniques such as solid-state NMR spectroscopy or cryo-electron microscopy (cryo-EM) confirmed the common architecture and shed light on their molecular organization [89].

Recent cryo-EM studies revealed remarkable details on the structure of fibrils extracted from AL patients [90–92]. In one structure (AL55), the fibril core is composed of 77 V_L domain residues (Fig. 2A) [90], while in another patient-derived fibril (λ1) the core consists of 91 V_L residues (Fig. 2B) [93]. In the third fibril structure depicted in Fig. 2 (FOR005), the core is made up of 115 V_L residues (Fig. 2C) indicating that the majority of the V_L domain is included. However, in both AL55 and FOR005 there are unresolved loops in the fibril structure, while in λ1 and FOR005 the N-terminal sequences are disordered (Fig. 2A,C) [90,92]. In all three V_L-derived fibrils, the intramolecular disulphide bond connecting the two β-sheets is preserved, while the rest of the native V_L structure is almost completely reorganized. Hence, the segments connected by the disulphide bond have reversed their orientation. This implies that substantial conformational transitions have to occur prior to forming the fibrillary structure. Morphing between the structures of the native V_L and the corresponding fibril illustrates the magnitude of structural changes that take place during the transition [93].

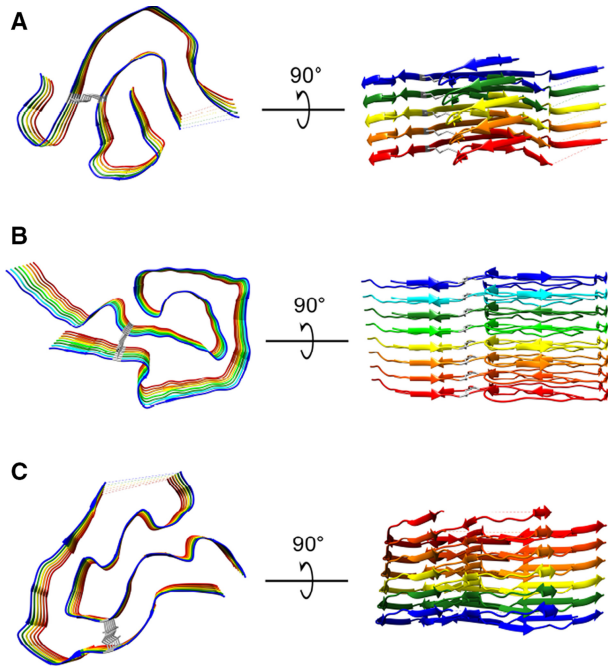

Fig. 2. Structures of amyloid fibrils from AL patients. Cryo-EM structures of (A) AL55 fibrils (PDB: 6HUD) and (B) λ1 fibrils (PDB: 6IC3). (C) FOR005 fibrils in conformation A (PDB: 6Z1O). In A, B and C, the different colors represent different polypeptide chains arising from V_L domains. The structures have been generated using UCSF Chimera.

The V_L fibrils show the typical cross-β structure stabilized by interactions along the fibril axis that include backbone hydrogen bonds and interactions between side chains [90–92]. However, each of the three fibril structures has a unique fold. The λ1 fibrils exhibit two channels that probably contain water molecules, while the other two fibrils lack this feature. Interestingly, FOR005 fibrils adopt two conformations within the fibril contributing to structural breaks. This finding demonstrates that fibrils from AL patients are not always conformationally uniform along the fibril axis [92].

Furthermore, an additional cryo-EM structure of a fibril (FOR001) was recently solved, identifying an N-glycosylation [94]. It had been suggested by several studies that glycosylation may be a risk factor for developing AL amyloidosis [95–97]. This seems to be a contradictory assumption at first since this post-translational modification normally leads to a stabilization of natively folded proteins [98]. However, in the case of patient LCs glycosylation was discussed to stabilize the fibril fold and protect it against proteolytic degradation [94]. How the additional glycosylation site in LCs affects their folding, unfolding and rearrangement to the fibril structure has to be investigated further.

The pathway of AL fibril formation

The observation that amyloidogenic proteins exist in two different stable states, the native state and the fibril, challenged the concepts of protein folding. It is believed that there is a considerable energy barrier between the two states. Thus, the native state needs to unfold to enter the fibrillary pathway and adopt the energetically more favorable amyloid state [99]. The molecular mechanism of how this barrier is overcome and which steps are involved are still not fully understood. A plausible explanation of fibril formation is

that an additional trigger combined with protein unfolding is needed which directs the protein towards fibril formation. This leads to the breakdown of the supersaturation barrier describing the phase transition from the soluble monomer to a crystal-like amyloid fibril structure [100,101]. These events take place in the comparably long and rate-limiting lag phase preceding fibril growth [101,102]. While fibril formation can be readily monitored by the binding of Thioflavin T, the events taking place in the lag phase cannot be captured by this approach. As mentioned above, the differences in the three-dimensional structures of the V_L domain and the fibril suggest that (partial) unfolding of LCs or their individual domains has to occur in the lag phase to initiate the fibrillary pathway (Fig. 2D). A conserved tryptophan residue in the V_L domain (Fig. 1D) can be used as a folding sensor, as its intrinsic fluorescence changes upon structural rearrangements and thus reports on conformational transitions in the lag phase. Recent results suggest that structural rearrangements occur already at the beginning of the lag phase and are accompanied by an increase in surface hydrophobicity [103]. These conversions lead to the formation of non-native dimers that seem to act as nuclei for oligomerization to hexamers and higher oligomers and finally fibril formation. These dimers differ from the physiological dimers that protect LCs from aggregation [104,105]. In the oligomeric state, a further structural transition occurs in which the typical parallel β-sheet structure found in fibrils is formed. These species represent the nuclei for fibril formation as fibrils rapidly emerge once the transition has occurred in the oligomers (Fig. 3) [103]. Of note, these conformational transitions seem to involve high energy barriers as they require destabilizing conditions such as mutations and long reaction times. An important open question that needs to be addressed in future research is how the structural transition propagates once the

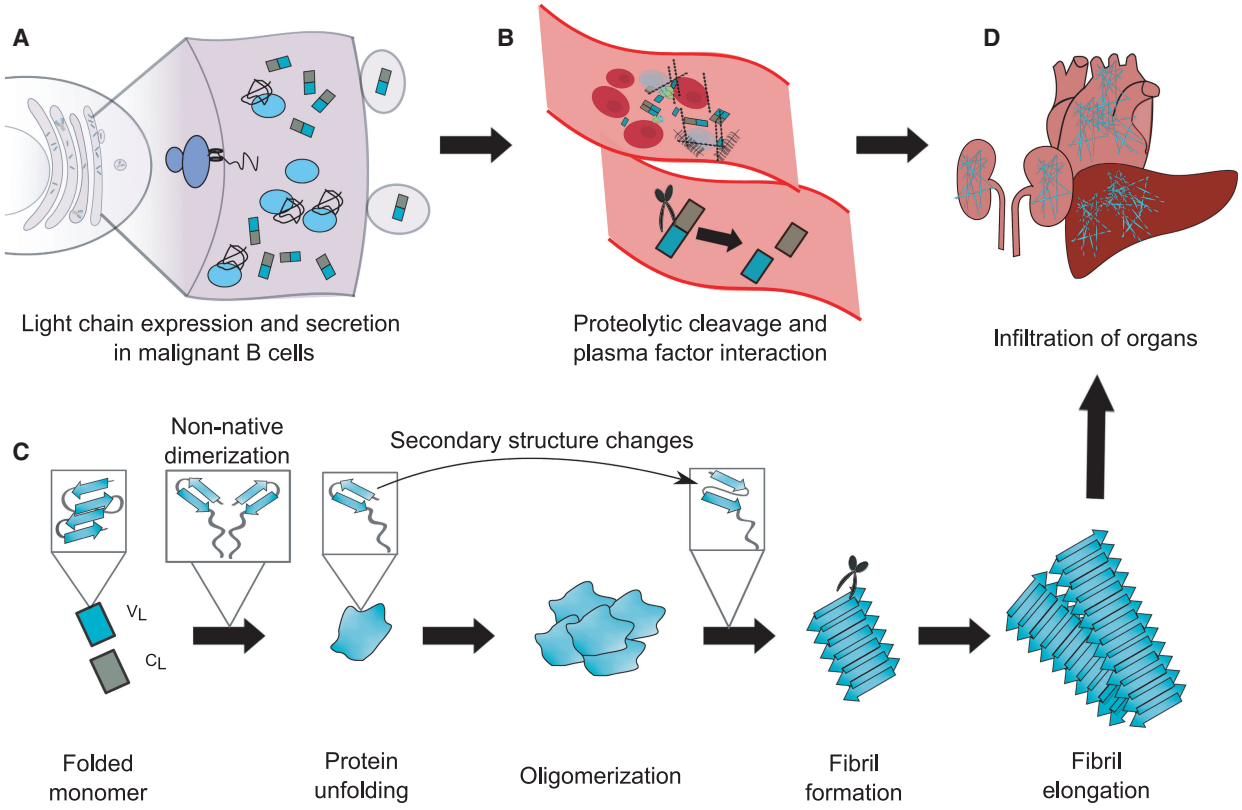

Fig. 3. AL amyloid formation pathway. (A) Mutant LCs are overexpressed and secreted by malignant B cells. The blow-up depicts the *ER*; blue ellipsoids represent ER chaperones, the squares are LCs. (B) LCs enter the bloodstream where proteolysis and interactions with plasma factors could occur; red ellipsoids are blood cells, grey ellipsoids represent other cell types, green circles show extracellular proteins and black lines are glycosaminoglycans. (**C**) To enter the fibril pathway, the LC or its V_L domain unfolds, dimerize and oligomerize followed by a secondary structure switch towards parallel β-sheets which leads to fibril formation and elongation. (D) The fibrils deposit in various organs and impair their function. Whether the proteolytic cleavage occurs in the bloodstream, in the organs or after fibril formation is still unclear.

fibril nucleus has formed. We still do not know whether the oligomerization of the monomers is still mandatory for elongation once a nucleus has formed or whether the interaction of partially unfolded LCs/V_Ls with the nucleus suffices for their structural rearrangement and fibril growth.

In the past, cytotoxicity in amyloid diseases was mainly attributed to the insoluble fibrils that affect organ function by depositing extracellularly in the tissue [20]. However, recent evidence suggests that the toxic effects could be exerted through intermediate, oligomeric species. These could disrupt membrane integrity by interfering with the phospholipids via their hydrophobic surfaces [106–108]. Furthermore, several other pathways for the pathological effects of the amyloid fibrils and their soluble precursors have been described. Extracellularly, cell viability is discussed to be compromised by the loss of cell–cell contacts due to fibril attachment [33]. Also, amyloid fibrils were shown to impair cell growth and division, while the soluble precursor proteins lead to dysfunctional cells that undergo apoptosis [33]. Moreover, both species seem to influence the functionality of mitochondria [34,109]. However, it is still an open issue which species and which process is pathologically most relevant.

The role of amyloidogenic point mutations

Often, the VJ rearrangement and SHM result in V_L domains with altered structural properties compared to the germline protein [110]. Together with the secretion of large amounts of free LCs into the bloodstream, this is the basis for subsequent steps leading to fibril formation [111]. A large number of different mutations that lead to AL amyloidosis have been identified and, accordingly, the changes they impose on the structural and biophysical properties of the LC and specifically the V_L domain can be very different. Hence, different mechanisms and pathways by which mutations promote amyloid aggregation of LCs seem to exist. According to present knowledge, amyloid-forming V_L domains contain approximately between four and fifteen mutations compared to their non-amyloidogenic germline counterpart sequence. However, often only a small fraction of these mutations is crucial for the destabilization and aggregation propensity of the domain [112–114]. To differentiate between so-called silent and active mutations, a detailed mutant analysis is necessary because the decisive substitutions are not always obvious [113,115]. Identification of amyloid-driving LC mutations is important since these data could allow clinicians to estimate the risk of developing AL amyloidosis for patients and the time frame of disease progression once the sequences of circulating free LCs (FLCs) are known.

In general, one can discriminate between the effects of mutations on the native fold of the LC/V_L domain or on the fibril structure. Therefore, LC mutations can be classified by whether their function lies in destabilizing and misfolding the soluble precursor protein or in stabilizing the final amyloid core structure – although it is of course also possible for a single mutation to serve both purposes. Mutations with an impact on the end product of the pathway, the insoluble amyloid fibrils, provide stabilizing interactions between stacked monomers along the axis of the protofilament or within the steric zipper and cross-β motifs inside the fibril core [93,116].

The best investigated and potentially most widespread effect of AL mutations is the thermodynamic destabilization of LCs and V_L domains. The connection between lowered thermodynamic stability and amyloid formation is well established and there are several examples in which amino acid substitutions drive misfolding and fibril formation by decreasing the unfolding free energy (ΔG_{un}) of the precursor protein (Table 1) [114,117–121]. Mutation of the highly-conserved residue R61 in patient-derived κ-sequences is a well-described case in which loss of the conserved salt bridge between E81/D82 and R61 leads to a strong destabilization of the V_L domain [119,122]. Accordingly, this mutation also affects the kinetic stability, that is, the rate constants of protein unfolding and refolding or, in other words, the rate-determining difference in free energy between the native and the transition state. The unfolding process of destabilized amyloidogenic V_L domains is accelerated and refolding is decelerated compared to non-amyloidogenic domains [112,123–125].

Mutations can also alter the thermodynamic properties of V_L domains or LCs by inhibiting refolding to the native state, thus rendering their unfolding irreversible [123,125,126]. However, this has also been reported for non-amyloidogenic Ig domains and it remains to be seen what additional features are responsible for reaching different final states [127].

A further mechanism by which mutations promote amyloidogenicity of V_Ls and LCs is the enhancement of protein backbone dynamics which is often accompanied by destabilization [128]. Various studies reported a link between amyloid formation and conformational flexibility [69,115,129,130]. Further, a connection between structural flexibility and cytotoxicity has also been proposed [131]. Dynamic fluctuations of the protein

Table 1. Selected examples of patient-derived AL sequences characterized by biophysical and/or structural methods. The mutations are annotated as the change from reference protein – so-called germline (GL) or MM sequence – to the patient sequence (PT). Mutations in bold have been shown to be strong drivers of amyloidogenicity compared to their germline counterparts. PDB accession codes for X-ray, NMR or cryo-EM structures are given. Different PDB codes for the same protein correspond to different structural methods used or to different crystallization conditions. This list is not comprehensive; a selection of sequences that represent the mechanisms of amyloidogenic mutations described above is shown. The asterisk next to MAK33 indicates that human mutations derived from the literature were introduced into a murine κ5 LC to study their effects on LC folding and stability.

Name [Ref.]	GL	Mutations (GL/MM → PT)	Biophysical/biochemical effects of point mutations	Structural data
3rJL2 [120,159]	λ3r	**P7D**, P8S, I48M, P40S, **W91A**	• The λ3r (= 3rJL2) germline is implicated in AL amyloidosis • Mutations P7D, I48M and W91A (CDR3) promote amyloidogenicity in λ3r • Mutations P8S and P40S inhibit fibril formation of λ3r • N-terminal region and loop 40–60 play a key role in stability and fibrillation	4AIX/4AJ0: λ3r
6aJL2 [17,121,129,130, 132,133,139,143,160]	λ6a	**F2X, P7S**, I29A, **R25G**	• The λ6a (= 6aJL2) germline is associated with the disease • Partially folded intermediate states play a key role in fibril formation • R25G appears in 25% of patients; it destabilizes 6aJL2 and boosts fibril formation ($\Delta\Delta G_{Un} = -1.7$ kcal·mol^{-1}) • Key role of mutations in N-terminal β-strand (Phe2, Pro7) • Dynamics and contact networks are important determinants of fibrillation	2W0K/2MMX: 6aJL2 5JPJ/2MKW: R25G mutant 3B5G/3BDX: P7S mutant
Mcg [68,147,161,162]	λ2	P14L, P42A, L48V, M49I, S54N, A94E, N97D	• As a reference, the λ2 germline sequence was derived from abYsis, IgBLAST and IMGT [163–165] • Fibril formation is initiated by V_L monomers → Dimer dissociation is the critical step in the amyloid formation of Mcg • LC adopts a different conformation when paired with another LC (= BJP) compared to regular pairing with the HC	1DCL/2MCG/3MCG: LC dimer 4UNU/4UNV: V_L dimer 4UNT: V_L monomer
WIL [117,134,146,166,167]	λ6	M3L, N5T, **D30A**, S31N, Q35H, Y50F, N53D, Q54H, A64S, I67V, **R69T**, A96H, R97N, V99Q, R106K	• Reference protein JTO is an LC similar to WIL from an MM patient • JTO carries mutations A30D and S69R with respect to the λ6 germline sequence; AL protein WIL contains Ala and Thr at these positions • D30 and R69 stabilize JTO compared to WIL via a salt bridge • Dimer dissociation is necessary for fibrillation, but the protective role of dimerization does not depend on the stability • CDR1 conformation of WIL differs from the canonical structure	2CD0: WIL 1CD0: JTO (MM)
AL-09 [126,148–152,168]	κ1	S30N, **N34I**, **K42Q**, N53T, D70E, I83L, **Y87H**	• Reference germline sequence: Vκ1 O18/O8 • Mutations induce rotated (90°) dimer interface (especially Y87H) • Tyr residues within the altered dimer interface modulate amyloidogenicity • Altered dimer conformation is linked to the formation of stable oligomeric intermediates	2Q1E: AL-09 2Q20: k1 germline V_L 2KQM: k1 Y87H 2KQN: AL-09 H87Y

Table 1. (Continued).

Name [Ref.]	GL	Mutations (GL/MM → PT)	Biophysical/biochemical effects of point mutations	Structural data
AL-12 [135,142]	κ1	S30T, **Y32H**, S65R, D70H, E81A, Q90E, N93Y, **Y96Q**, Q100G, L104V	• Reference germline sequence: Vκ1 O18/O8 • Subtle conformational changes occur in the Pro40 loop and CDR3 loop but the canonical dimer interface is intact • H32 and Q96 are key residues in amyloid formation • Fibrillation is not connected to stability difference but rather to the sampling of partially folded states	3DVF: AL-12 2Q20: κ1 germline V_L
AL-103 [112,135]	κ1	N34I, D92H, **P95a-inserted,** Q101P	• Reference germline sequence: Vκ1 O18/O8 • Small structural changes in the CDR3 loop, but canonical dimer interface • Stability and fibril formation are affected by Pro insertion in CDR3 (P95a) • Deletion of P95a enhanced stability of AL-103 and delayed fibril formation • Suggested influence of cis/trans prolyl isomerization on amyloidogenicity	3DVI: AL-103 4K07: P95a deletion 2Q20: κ1 germline V_L
SMA [105,114,138,141,169]	κ4	S29N, K30R, **P40L,** Q89H, T94H, **Y96Q,** S97T, I106L	• Reference protein LEN is an LC similar to SMA from an MM patient • Mutations (esp. P40L and Y96Q) destabilize SMA compared to LEN and induce fibrillation ($\Delta\Delta G_{Un}$ = −2.6 kcal·mol^{-1}) • Partially folded intermediates play a key role in the amyloid pathway • Different intermediate states can enter different pathways, i.e., amyloid or amorphous aggregation	1LVE: LEN (MM)
Rec [114,169]	κ4	**L15P**, S27aN, V27bL, Y27dD, S27eA, **N28F**, S29D, **K30T**, Y32T, T53S, **Y96P**, S97T, Q100G, L104V	• AL-protein Rec is destabilized ($\Delta\Delta G_{Un}$ = −4.4 kcal·mol^{-1}) in comparison to MM-protein LEN • L15P, N28F, K30T and Y96P destabilize and induce fibril formation when introduced into LEN • Mutations N28F and K30T are located in CDR1, Y96P is located in CDR3 → Stability and amyloid formation can be modulated by mutations in hypervariable CDR loops	1LVE: LEN (MM)
BRE [170,171]	κ1	I30S, K31D, **N34I,** T39K, **P40L**, **K45N, E50D**, N53T, Q55E, A56T, D70E, Q92D, S93D, L104V, Q105E, T107K	• Reference protein REI is an LC sequence from an MM patient • BRE is strongly destabilized compared to REI (ΔT_m = −20.4°C) • Mutations N34I and P40L induce an altered dimer interface • Mutations K45N and D50E alter surface properties and modify the electrostatic surface potential of BRE	1BRE/1B0W/1QP1: BRE 1REI: REI (MM)
Pat-1 [113]	λ2	P15L, T26S, S28N, V30F, G32D, E53D, S55D, **L81V,** Q82L, Y90F, T105S	• The λ2 germline sequence was determined with abYsis, IgBLAST and IMGT [163–165] • Mutations (esp. L81V) disrupt a hydrophobic interaction network in the V_L domain → Decrease in stability of patient V_L compared to germline V_L and induction of fibril formation (ΔT_m = 8.1°C) → Enhanced dynamics in C-terminal segment of the domain	6SM1: Germline V_L 6SM2: Pat-1

Table 1. (Continued).

Name [Ref.]	GL	Mutations (GL/MM → PT)	Biophysical/biochemical effects of point mutations	Structural data
FOR005 [115,116,172]	λ3	Y31S, Y48F, N51S, **G49R**, **G94A**	• The λ3 germline sequence was determined with abYsis, IgBLAST, IMGT [163–165] • CDR mutations (esp. G94A) increase dynamics in conserved framework regions and decrease stability • G49R destabilizes V_L and also stabilizes the fibril core • Fibril formation correlates with a deviation from canonical CDR classes	5L6Q: FOR005 6Z1O/6Z1I: Cryo-EM of *ex vivo* fibril
MAK33* [71,122,157]	κ5	**S20N** [173], D70N [174], **R61A** [119]	• S20N/D70N: stability is not impaired, but fibrils form due to non-native interactions involving Asn residues • R61A: loss of salt bridge between R61 and E81/D82 destabilizes V_L • V_L–C_L interactions and linker region determine amyloidogenicity of the LC	1FH5

Bold formatting indicates mutations that strongly favor amyloid formation.

backbone are a complex feature that requires sophisticated analytical methods including NMR spectroscopy, hydrogen/deuterium exchange, limited proteolysis and molecular dynamics simulations. Importantly, a larger number of sequence variants needs to be studied to gain more detailed insights into the connection between protein dynamics and amyloid aggregation.

The effects imposed on the V_L domain by a mutation depend on its specific position [120,132,133]. Destabilizing effects of non-conservative mutations in the conserved framework regions are mostly straightforward to rationalize [119]. However, it has also been shown that patient-specific substitutions in exposed, hypervariable CDR loops can induce structural changes or enhance backbone dynamics which render the domain amyloidogenic [112,115,134–136].

Additional factors that play a key role in AL amyloidosis are the population of partially folded intermediate states and transient, non-native interactions which allow assembly into amyloid fibrils [105,137–139]. In comparison to their non-amyloidogenic counterparts, these variants more readily transition into a conformation that differs from the native state [71,140,141]. Certain point mutations in the V_L domain can promote the transition into these aggregation-prone folding intermediates [142,143]. Yet, these mutations do not necessarily destabilize the native state of the V_L domain or LC. It has been reported that amino acid substitutions which hardly alter the thermodynamic stability of a native LC or V_L domain can cause the formation of amyloid fibrils by inducing specific non-native interactions which favor the amyloid state [122]. In other words, the transition barrier to the amyloid state is lowered without affecting that between the native and unfolded state.

The quaternary structure of the precursor is another important aspect of LC amyloid aggregation. It is well established that dimerization can protect LCs and V_Ls from misfolding and aggregation [144–146]. The dissociation of LC–LC or V_L–V_L homo-dimers into a monomeric state is often the first step of the fibrillar pathway [147]. Accordingly, mutations that change the dimer interface or disrupt interactions within the interface are associated with decreased stability and amyloidogenesis [148–152]. Preventing fibril formation by stabilizing LC–LC or V_L–V_L dimers has been proposed as a novel therapeutic approach [153]. However, while this approach is promising, it should be noted that not all LCs and V_Ls form dimers in solution and that also secretion of monomeric LCs into the blood stream has been reported [153–156].

Furthermore, it is important to distinguish between the effects of mutations on V_L domains and full-length LCs as they can be quite different [124]. Intuitively, one may assume that linking a destabilized V_L domain to a stable C_L domain protects full-length LCs from amyloid aggregation whereas the corresponding single V_L domain forms fibrils *in vitro*. This has indeed been demonstrated for several examples [113,115]. However, it has also been shown that isolated V_L domains can be thermodynamically more stable than the respective full-length LC. Thus, the interactions and orientation between the V_L and C_L domains in the LC are

important determinants of the biophysical properties and the aggregation propensity of LCs [70,71,157]. Further, the role of proteolytic cleavage of LCs which yields aggregation-prone single V_L domains is also connected to the structural features of the full-length LC [125,158].

Proteolytic cleavage of LCs and fibrillar structures

Mutations also seem to play a role in the susceptibility of LCs to proteolysis [175]. There is still a debate about how important proteolysis is for AL amyloidosis. Generally, the decreased stability and partial unfolding of the antibody LCs seem to make them more accessible to endoproteolysis, as was shown by *in vitro* experiments with recombinant LCs [69,175]. The fragmentation pattern of secreted LCs in fibrils was determined after extracting them from patient tissues. The results suggest that some parts of the LC seem to be more prone to proteolysis than others (Fig. 4) [90,93,113,176,177]. Particularly, the linker connecting the V_L and C_L domains seems to be affected. Several studies showed that both the complete LCs and fragments thereof, as well as the variable and constant domains, can be deposited [113,176–179]. However, it also became clear that C-terminally truncated LCs and especially the V_L domain are mainly found in the central core of the fibril [113,176,177,180]. Together with *in vitro* experiments this suggests that predominantly the V_L domain is amyloidogenic and underpins the necessity of proteolytic cleavage. However, amyloid deposits in some patients can contain full-length LCs or even C_L domains [176,181,182], challenging the 'V_L-model' for AL amyloidosis. Although it is well established that proteolysis of mutant LCs occurs *in vivo*, there is still an ongoing debate about the timing of proteolysis. Whether it precedes amyloid formation and takes place in the bloodstream or amyloids form first and are subsequently proteolytically cleaved in the tissue is still not clear. There are studies that support either of these theories and a clear conclusion cannot be drawn yet [113,158,177,180]. The different and contradictory data imply that the occurrence and timing of proteolysis could be a case-dependent phenomenon. In cases where the C_L domain exerts a protective function, mutations could promote proteolysis of the full-length LC thereby enabling aggregation of a destabilized V_L.

Besides the timing of proteolysis, the protease(s) responsible for the cleavage(s) of the isolated LC or the fibrils have not been identified yet. However, some candidates have already been associated with AL proteins as summarized in Table 2. The cysteine proteases Cathepsin (Cath) K, L and B, normally active in lysosomes, are produced by macrophages and multinucleated histiocytic giant cells. They are major factors for tissue and extra cellular matrix (ECM) remodelling and are often co-localized with AL deposits [183]. Also, CathK, L and B were shown to degrade AL fibrils from tissue sections and monomeric recombinant AL proteins [183,184]. Since the degradation was highly pH- as well as concentration-dependent and since Caths are only stable to a limited extent in the extracellular milieu, the actual role of CathK, CathL and CathB in amyloid degradation *in vivo* is still not solved. In addition to Caths, matrix metalloproteases (MMPs) and their inhibitors (TIMP) seem to influence AL *in vivo*. While in cardiomyopathy an increase of the serum

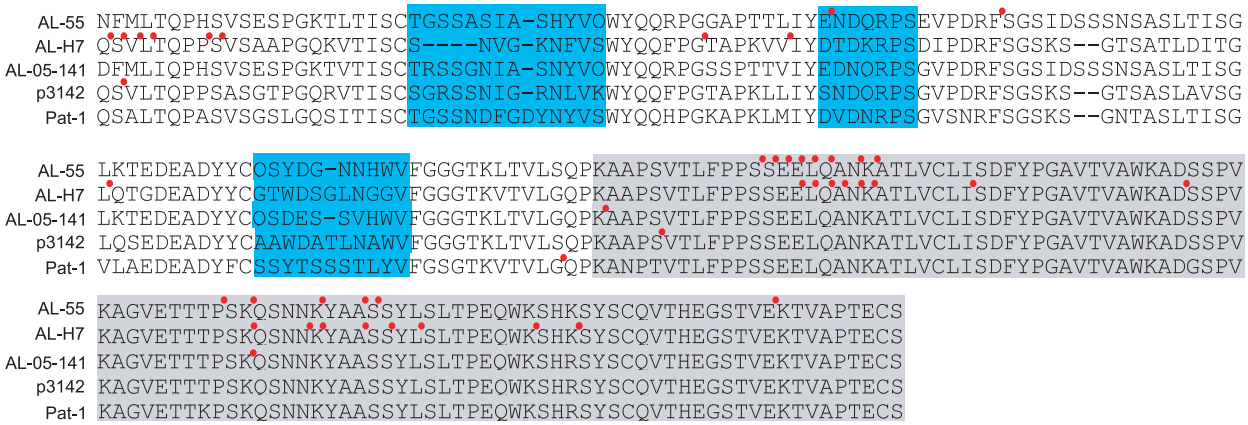

Fig. 4. Proteolysis sites in antibody LCs found in AL deposits. Alignment of the five patient-derived LC sequences AL-55 (PDB: 6HUD), AL-H7 (PDB: 5MUH) [177], AL-05-141 (no PBD entry available) [176], p3142 (PDB: 6IC3) [93] and Pat-1 (PDB: 6SM2) [113] using clustalo 1.2.4. The respective proteolytic cleavage sites are shown as red dots. CDRs are highlighted in cyan (according to the Kabat numbering), the C_L domain is in grey.

Table 2. Proteases potentially associated with AL amyloidosis. Plasma concentrations were adopted from the human proteome atlas (https://www.proteinatlas.org/) unless otherwise stated.

Protease	Function	Plasma concentration	Publication
Cath B	Intracellular protein degradation and extracellular cleavage	130 $\mu g \cdot L^{-1}$	[184]
Cath K	Extracellular matrix degradation and limited proteolysis	Not applicable	[183]
Cath L	Probably degradation of proteins in the lysosome, limited proteolysis of	17 $\mu g \cdot L^{-1}$	[184]
MMP-1	Cleaves various collagen types	0.43 $\mu g \cdot L^{-1}$	[187, 190]
MMP-2	Ubiquitous, involved in tissue remodelling	160 $\mu g \cdot L^{-1}$	[187,190]
MMP-7	Degradation of casein, gelatins and fibronectin. Activates procollagenases	3.25 $\mu g \cdot L^{-1}$	[190,191]
MMP-9	Essential for extracellular matrix proteolysis and leukocyte migration, cleaves type IV and V collagen	57 $\mu g \cdot L^{-1}$	[186,187,190]
TIMP-1	Inhibitor of various MMPs; growth factor	750 $\mu g \cdot L^{-1}$	[186,187]

levels of MMPs and TIMPs were correlated with poor prognosis [185,186], in cases with renal involvement a different picture emerged. There, MMP but not TIMP levels appeared to be generally elevated in AL amyloidosis and a co-deposition of TIMP with AL fibrils was shown [187]. So far, no specific conclusion on the involvement of metalloproteases in AL can be drawn but an organ-dependent behaviour needs to be considered. Direct cleavage of AL proteins by MMPs has not been shown yet. However, proteolytic activity against other amyloidogenic proteins has been demonstrated [188]. For serine proteases, only little is known in the context of AL amyloidosis, although in other amyloid diseases amyloid deposits can be cleared by the plasmin system [189]. Despite efforts to understand the mechanism of fibril formation in association with proteolytic cleavage, it is still not clear which species is primarily responsible for fibril formation and at which time point the proteolytic cleavage occurs. Also, the role of co-deposited proteases in the extracellular matrix needs further investigation to determine whether they serve as amyloid degrading components or if, in contrast, they create pathogenic species. Furthermore, in addition to co-deposited proteases, an interaction with circulating proteases in the bloodstream is also possible. But since these interactions are more transient and may not be detectable once fibrils have already formed, efforts have to be made, to identify which proteases are involved. This is further complicated by the possibility that several exo- and endo-proteases could be participating in the processing of an AL protein (Fig. 4) [177].

ER quality control and LC escape

The endoplasmic reticulum (ER) is a highly optimized compartment for protein folding and maturation of proteins that are destined for membrane integration, transfer to organelles or secretion. Plasma cells are specialized for the production and secretion of massive amounts of antibodies: one plasma cell secretes about 1000 antibodies per second [192]. Accordingly, they possess an expanded ER to cope with the folding load [193]. During the folding process, nascent polypeptides undergo a number of processing steps like disulphide bond formation or N-linked glycosylation. The ER quality control (ERQC) system monitors the proteins regarding their correct folding and assembly and directs aberrant chains to the ER-associated degradation (ERAD) system which leads to proteasomal degradation [194]. These processes require assistance from a range of molecular chaperones and other folding factors. As each antibody molecule needs chaperone assistance to guarantee correct processing and assembly before secretion is allowed, an imbalance in the ratios of folding factors and folding proteins leads to ER stress and triggers the so-called unfolded protein response (UPR). The UPR reduces general protein synthesis while at the same time enhancing the expression of ER-specific folding factors [195].

Light chains are naturally produced in excess compared to HCs [192]. Under physiological conditions, the ERQC ensures that only assembled antibodies are secreted due to specific chaperone-based control mechanisms involving the retention of free HCs in the cell [196]. The secretion control is not as strict for LCs. Thus, small amounts of secreted LCs are a common byproduct of antibody synthesis in healthy individuals [197–199]. However, the LC level is highly elevated in diseases like monoclonal gammopathies, MM, LC deposition disease (LCDD) and AL amyloidosis [200–202]. Due to unknown reasons, these diseases differ in many features like predominant pathogenic LC subtype (λ or κ), phenotype and deposition of LCs, suggesting different mechanisms of pathogenicity. In AL amyloidosis, the high serum level of mutated LCs in patients is a major determinant [203]. Since in many

cases, the patient LCs are destabilized by mutations, one would assume that they are detected as misfolded or non-native by the ERQC. Obviously, this is not the case. At present, one can only speculate why they are not recognized.

Secretion of proteins is overall dictated by their ability to fold correctly and by their thermodynamic and kinetic stability [204]. Consequently, the LCs need to be initially folded to be secretion-competent. The presence of un- or misfolded proteins in the ER would lead to their recognition by molecular chaperones and co-chaperones like binding Ig protein and ER DnaJ homologues [205]. Proteins unable to fold in the ER are targeted for ERAD [206–209]. In this regard, crystal structures of patient-derived amyloidogenic LCs and LC truncations confirm the ability of these variants to fold correctly into the typical Ig fold [69,113,172]. There are several conceivable mechanisms of how LCs can successfully escape ERQC. The first is that the huge overload of synthesized LCs leads to an imbalance that cannot be compensated by UPR pathways of increasing folding and degradation levels [210]. Besides, monomeric LCs possess a free thiol group that is usually recognized as part of immature proteins by the ERQC [211]. Furthermore, LC dimerization could play a role. In AL amyloidosis, LCs of λ subtype are predominantly the cause for disease development. These LCs are more prone to dimerization than κ LCs and this could mask their pathogenic features [212–214]. In general, we still know little about the biosynthesis of amyloidogenic LCs. This is illustrated nicely by the fact that the mutation of the conserved residue R61 and the resulting missing salt bridge, which has already been found in patients and studied *in vitro* [119,122], was retained by the cell machinery in another study [156].

Interaction of amyloidogenic FLCs with extracellular components

A defining trait of AL amyloidosis is that the constituent LCs are secreted in high levels to the extracellular space [215–217]. In the bloodstream, they are exposed to factors, which could affect their stability and integrity [218]. These include chaperones, ECM proteins, glycosaminoglycans and lipids.

Extracellular chaperones bind partially unfolded and aggregation-prone proteins to prevent further aggregation. Eventually, these complexes may be taken out of circulation via endocytosis. These extracellular proteostasis mechanisms have been recently reviewed [219]. In the extracellular fluids, the ubiquitous chaperone Clusterin [Apolipoprotein (Apo) J] seems to be one of the major factors to maintain protein homeostasis [220,221]. In AL amyloidosis, Clusterin is one of the proteins that co-deposit with the fibrils [222]. It was shown that Clusterin can bind amyloid proteins and facilitate their lysosomal degradation after endocytosis [223]. Thus, a beneficial effect on disease development and progression can be envisioned.

Among the plasma proteins co-deposited in AL amyloidosis, as identified by mass spectrometry-based approaches, there are also serum amyloid component P (SAP), Vitronectin and Apo E (ApoE) [224,225]. SAP binds to amyloid structures in a calcium-dependent manner and serves as a biomarker in AL diagnostics [226–228]. Its role is not fully understood, however, it was shown that SAP prevents AL amyloid deposits from proteolytic degradation [229]. Apo E is an important factor for lipid transport. It is considered to be a risk factor for Alzheimer's disease but, besides its co-deposition with fibrils, its influence on AL amyloidosis is unknown [230,231]. In addition to protein components, polysaccharides were shown to co-deposit with fibrils and to enhance fibril formation *in vitro* [232,233]. The possible mutual stabilizing function through salt bridges of negatively charged polysaccharides and accessible basic residues in the fibrils has been discussed recently [234]. Whether the proteins, glycosaminoglycans and lipids present in fibrillar deposits are a consequence of fibril formation, a reason for it or both remains to be clarified. Even if the functions of the factors described above have not yet been fully resolved, a signature of factors in the deposits that is specific to amyloidosis emerges.

Conclusions and perspectives

As described above, AL amyloidosis is a very special disease, which is also set apart from another amyloidosis by the large number of patient-specific mutations associated with it. Currently, it is not yet clear which of these numerous mutations identified in patient proteins are really linked with the disease. Progress made in recent years suggests that only a few of these mutations play an active role in promoting fibril formation. However, only some patient variants have been investigated in greater detail in this context. The emerging picture from these studies is that most 'active' mutations destabilize the V_L domain and this allows accessing the fibrillary pathway. Interestingly, this can be achieved by mutations at different positions of the framework or in the CDRs. Clearly, a larger number of well-characterized patient mutations is needed to achieve a comprehensive picture. We envision that in this process, additional mechanisms for shifting LCs to the amyloid state will be discovered. An example are

mutations that specifically stabilize the fibrillary fold as in the fibrils the native state is completely rearranged. Together, the analysis of mutants on the one hand will result in a detailed understanding of the disease on the molecular level. On the other hand, it will also provide novel insights into the principles of protein folding and stabilization, as the amyloid LC variants represent a 'library' of sequences that fold into the Ig structure but have lost specific yet unknown features that normally keep amyloid formation at bay. Furthermore, determining the effects of plasma factors on fibril formation of V_L variants will provide a deeper understanding of the amyloid pathway *in vivo* with a view to identifying novel sites for therapeutic intervention. In this context, another point of attack is the amyloid precursors. As the LCs have to unfold, chemical compounds binding to the native state could block the fibrillary pathway at an early stage [118]. That this is possible in principle has been shown already [54]. Whether proteolysis could also be a step at which one could intervene with amyloid formation therapeutically remains to be seen – as it is still unclear when along the pathway cleavage occurs and which proteases are involved.

In our view, the progress made in recent years has paved the way for defining a molecular framework of AL amyloidosis which allows us to tackle the key open questions in the near future. The continued collaboration of clinicians and basic scientists will be required to connect these findings to diagnosis and treatment with a view to making AL amyloidosis a curable disease.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

RMA, GJR, HLS, PK and JB wrote the manuscript. RMA and HLS created the figures, GJR has compiled the overview of investigated LC mutants.

Data availability statement

All data are contained within the article.

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